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Identification of a Novel GB Type C Virus/Hepatitis G Virus Subtype in Patients With Hematologic Malignancies

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The existence of four GB C virus/hepatitis G virus (GBV-C/HGV) subtypes has been reported. The subtype was determined in 16 multitransfused GBV-C/HGV infected patients prior to bone marrow transplantation by comparing the 5′ untranslated region (5′ UTR) sequence with 39 available sequences. Phylogenetic and bootstrap analyses were carried out with PHYLIP package 3.5c. In the samples with undefined subtype, the whole 5′ UTR was cloned and sequenced. Comparison of distances showed that the isolates from 12/16 and 4/16 patients belonged theoretically to subtypes 2a and 2b, respectively. The phylogenetic tree and bootstrap analyses confirmed this result in only 11/16 samples. Analysis of the entire 5′ UTR from the remaining five samples with undefined GBV-C/HGV subtype revealed genomic variability within the isolates from each patient and between the isolates of different patients. Evolutionary distances, phylogenetic tree, and bootstrap showed that the isolates from these samples were grouped in a separate branch, different from the published subtypes. In conclusion, a novel GBV-C/HGV subtype was found in a group of multitransfused patients with GBV-C/HGV infection. ***J. Med. Virol. 57:80–84, 1999.***

KEY WORDS: bone marrow transplantation; multitransfused patients; phylogenetic analysis

INTRODUCTION

Recently, a novel hepatitis-associated agent termed GB C virus (GBV-C) was isolated [Simons et al., 1995a]. Thereafter, Linnen et al. [1996] identified another agent that was named hepatitis G virus (HGV). Sequence analysis of the GBV-C and HGV genomes revealed that both agents were two isolates of the same virus, which belongs to the *Flaviviridae* family [Leary et al., 1996]. GBV-C/HGV genome is a positive-strand RNA molecule of approximately 9.4 Kb in length [Leary et al., 1996]. Analysis of GBV-C/HGV RNA demonstrated that the genomic organization shares characteristics common to those of the other flaviviruses such as hepatitis C virus (HCV) [Simons et al., 1995b]. Thus, the genomes of both viruses have an untranslated region located at the 5′ end of the RNA molecule (5′ UTR), preceding a long open reading frame that codifies for a single polyprotein [Leary et al., 1996].

Phylogenetic analysis of the 5′ UTR nucleotide sequence revealed the existence of GBV-C/HGV subtypes with different prevalence in distinct areas, as occurs with HCV [Fukushi et al., 1996; Muerhoff et al., 1996, 1997; An et al., 1997]. To date, four GBV-C/HGV subtypes have been identified: subtype 1 isolated in west Africa, subtypes 2a and 2b in Europe and the United States, and subtype 3 in Asia [Fukushi et al., 1996; Muerhoff et al., 1996, 1997; An et al., 1997].

The main GBV-C/HGV infection acquisition route is parenteral exposure, as demonstrated by the high prevalence of this agent in both transfused patients and in patients who have undergone bone marrow transplantation (BMT) [Linnen et al., 1996; Rodríguez-Iñigo et al., 1997; Tomás et al., 1997]. As BMT patients are exposed to a large number of different blood donors before BMT, it is likely that they may be infected by many different GBV-C/HGV strains. However, to date, the distribution of GBV-C/HGV subtypes has not been studied in these patients. In this study the GBV-C/

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TABLE I. Mean of Evolutionary Distances Between the 320-bp 5′ UTR Sequence Obtained From Each Patient and the 5′ UTR Published Sequences From Each GBV-C/HGV Subtype

	GBV-C/HGV subtype[a]			
Sample	1	2a	2b	3
S1	0.1514 (0.1419–0.1635)	0.0725 (0.0599–0.0894)	0.0898 (0.0842–0.1044)	0.1280 (0.1253–0.1306)
S2	0.1541 (0.1419–0.1652)	0.0623 (0.0503–0.0793)	0.0782 (0.0695–0.0892)	0.1172 (0.1146–0.1198)
S3	0.1379 (0.1270–0.1535)	0.0473 (0.0363–0.0599)	0.0567 (0.0503–0.0744)	0.0920 (0.0894–0.0945)
S4	0.1343 (0.1208–0.1482)	0.0457 (0.0316–0.0600)	0.0615 (0.0551–0.0793)	0.0970 (0.0944–0.0995)
S5	0.1301 (0.1208–0.1429)	0.0395 (0.0270–0.0552)	0.0567 (0.0503–0.0744)	0.0920 (0.0894–0.0945)
S6	0.1442 (0.1255–0.1646)	0.0379 (0.0270–0.0599)	0.0499 (0.0409–0.0647)	0.0919 (0.0893–0.0944)
S7	0.1337 (0.1152–0.1539)	0.0310 (0.0179–0.0551)	0.0451 (0.0363–0.0599)	0.0820 (0.0795–0.0845)
S8	0.1328 (0.1201–0.1482)	0.0317 (0.0225–0.0457)	0.0356 (0.0225–0.0551)	0.0769 (0.0744–0.0793)
S9	0.1278 (0.1049–0.1478)	0.0302 (0.0179–0.0457)	0.0358 (0.0270–0.0504)	0.0722 (0.0697–0.0746)
S10	0.1249 (0.1101–0.1422)	0.0271 (0.0179–0.0503)	0.0426 (0.0363–0.0598)	0.0769 (0.0744–0.0794)
S11	0.1474 (0.1314–0.1655)	0.0420 (0.0272–0.0648)	0.0547 (0.0457–0.0697)	0.0820 (0.0795–0.0845)
S12	0.1306 (0.1152–0.1482)	0.0263 (0.0134–0.0456)	0.0379 (0.0317–0.0551)	0.0623 (0.0599–0.0647)
S13	0.1261 (0.1099–0.1425)	0.0383 (0.0270–0.0503)	0.0154 (0.0089–0.0316)	0.0623 (0.0599–0.0647)
S14	0.1213 (0.1051–0.1377)	0.0332 (0.0225–0.0457)	0.0099 (0.0044–0.0225)	0.0624 (0.0600–0.0648)
S15	0.1450 (0.1203–0.1642)	0.0473 (0.0363–0.0599)	0.0384 (0.0317–0.0551)	0.0720 (0.0695–0.0744)
S16	0.1451 (0.1257–0.1592)	0.0596 (0.0409–0.0697)	0.0364 (0.0317–0.0552)	0.0844 (0.0844–0.0844)

[a]The numbers in parentheses indicate the range of the observed evolutionary distances.

HGV subtype was determined in 16 multitransfused patients with GBV-C/HGV infection before BMT.

MATERIALS AND METHODS

Serum samples from 16 multitransfused patients (5 males and 11 females) with hematologic malignancies and a mean age of 34.0 ± 13.6 years were included in this study. All patients had serum GBV-C/HGV RNA. The diagnosis in 11 patients was acute myeloid leukemia, 4 had acute lymphoblastic leukemia, and 1 had non-Hodgkin's lymphoma. All samples were taken prior to BMT (mean day prior to BMT: day −5; range: −1 to −16). Before BMT, the patients had received a large number of blood transfusions (mean of exposed donors: 94.7 ± 83.1). They had mean alanine aminotransferase levels (ALT) of 55.8 ± 55.9 IU/l (11 patients had normal ALT levels and the remaining 5 had abnormal ALT levels). None of the patients had hepatitis B surface antigen and two had antibodies against HCV and serum HCV RNA.

Total RNA was extracted from 250 μl of serum using Trizol LS reagent (GIBCO-BRL, Bethesda, MD) and chloroform, followed by isopropanol precipitation at −80°C. The RNA pellet was resuspended in 20 μl of RNase-free distilled water and 5 μl were used for GBV-C/HGV amplification. Reverse transcriptase–nested polymerase chain reaction (RT-PCR) of the GBV-C/HGV 5′ UTR was carried out as previously described [López-Alcorocho et al., 1997]. The RT-PCR products (320 bp) were purified from 1.5% agarose gels using the QIA quick Gel Extraction kit (Qiagen, Hilden, Germany) and automatically sequenced in an ALF express system (Pharmacia Biotech, Uppsala, Sweden).

The nucleotide sequences were aligned with the CLUSTAL W program, version 1.6 [Thompson et al., 1994], and compared with 39 5′ UTR sequences obtained from GenBank: 16 from subtype 1 (U59540–U59555), 12 subtype 2a (U59518–U59528 and U44402), 9 subtype 2b (U59529–U59537), and 2 subtype 3 (U59538 and U59539). Phylogenetic analysis was carried out with the PHYLIP package version 3.5c [Felsenstein, 1989]. Evolutionary distances were estimated with the Kimura 2-parameters method using the DNADIST program (PHYLIP) and unrooted trees were constructed by the UPGMA method using the NEIGHBOR and DRAWGRAM programs (PHYLIP). Bootstrap analysis of 1,000 replicates was undertaken with the SEQBOOT, DNAPARS, and CONSENSE programs (PHYLIP). When the bootstrap value is less than 70%, it was considered that no evidence exists for the phylogenetic grouping observed.

In the samples with undefined genotype, the nucleotide sequence of the entire 5′ UTR was analyzed. Thus, total RNA from sera was extracted as described above and amplified by heminested RT-PCR, using degenerated primers deduced from all the GBV-C/HGV 5′ UTR published sequences. The reverse transcription and first amplification round were carried out with the sense primer US1: 5′-CACTGGGTGCAAGCCCCA-3′ (nucleotides 1–18) and the antisense primer UA1: 5′-TTCCTCTCAAAYTGYTGYGCYYYRGA-3′ (nucleotides 611–636). The second round of PCR was carried out with the sense primer US1 and the antisense primer UA2: 5′-CCCCGGCCACMCAYGCTTCYMG-AGC-3′ (nucleotides 573–596). The reverse transcription and heminested PCR conditions were the same as those described previously [López-Alcorocho et al., 1997]. The nucleotide position of the primers is given according to the GBV-C nucleotide sequence reported by Erker et al. [1996]. The PCR products (596 bp) were cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Several clones from each sample were sequenced automatically and the nucleotide sequences were analyzed as described above. The GenBank Accession Numbers of the sequences reported in this article are AF038780–AF038814.

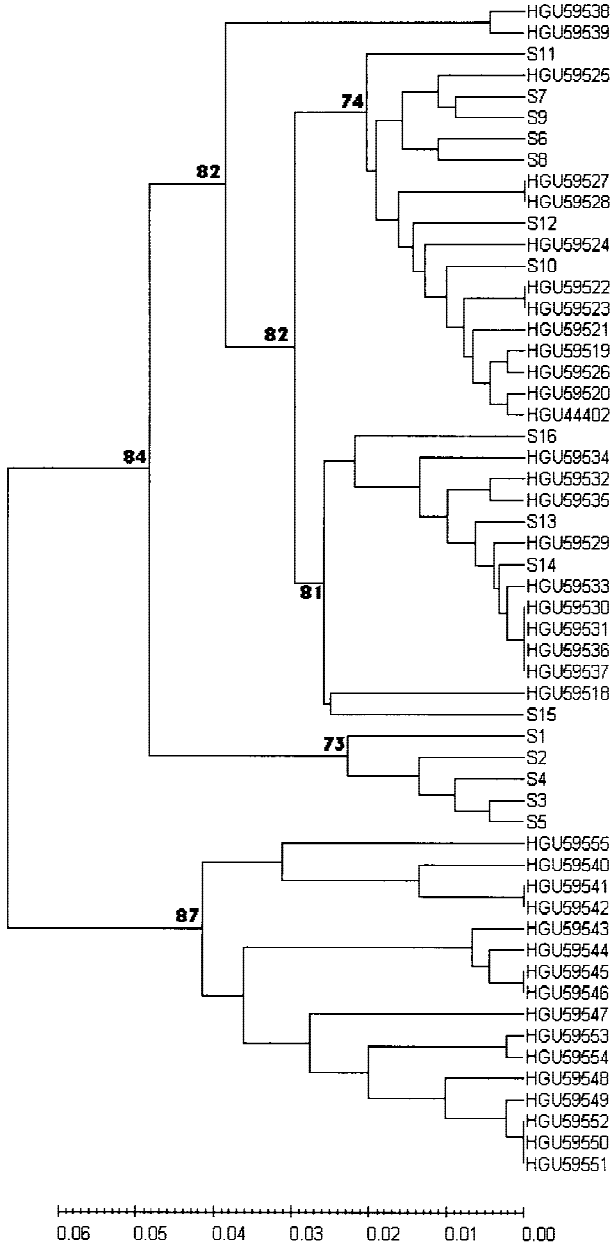

Fig. 1. Unrooted phylogenetic tree constructed with the GBV-C/HGV 5′ UTR sequences isolated from the 16 patients included in this study (S1–S16) and the available published sequences of each GBV-C/HGV subtype (subtype 1: HGU59540–HGU59555; subtype 2a: HGU59518–HGU59528 and HGU44402; subtype 2b: HGU59529–HGU59537; and subtype 3: HGU59538 and HGU59539). The bootstrap values are indicated in the tree nodes.

RESULTS

Comparison of the pairwise evolutionary distances of the 320 bp 5′ UTR PCR products of the GBV-C/HGV isolated from the 16 patients included in this study showed a high degree of homology among all the nucleotide sequences. Thus, the mean evolutionary distance among all the isolates from these patients was 0.0494 (range: 0.0089–0.1096). In order to assign each isolate from the patients to one of the described GBV-C/HGV subtype, the nucleotide sequences were compared with the published 5′ UTR sequences from each subtype. Based on the genetic distances between 320-bp 5 UTR PCR products from each patient and the 5′ UTR published sequences from each subtype, the GBV-C/HGV isolated from 12/16 (75%) patients theoretically belonged to subtype 2a, while that from the remaining 4 patients (25%) belonged to subtype 2b (Table I). The phylogenetic tree confirmed that the four samples mentioned had sequences belonging to subtype 2b. However, only 7/12 sequences, which at first were classified as subtype 2a, grouped together the 2a sequences published. The remaining five sequences, previously classified as subtype 2a, segregated into a separate branch, although they were closely related with the sequences of type 2 (Fig. 1). The phylogenetic tree was supported strongly by bootstrap analysis of 1,000 replicates. Thus, these five sequences segregated into a separate branch with a bootstrap value of 73%.

TABLE II. Evolutionary Distances Between the Different Clones From the Entire GBV-C/HGV 5′ UTR Nucleotide Sequence

Sample	Number of clones analyzed	Mean of distances	Range
S1	4	0.0065	0.0000–0.0165
S2	5	0.0078	0.0000–0.0131
S3	4	0.0104	0.0033–0.0164
S4	5	0.0171	0.0000–0.0366
S5	1		

To analyze further whether these five isolates belonged to or did not belong to one of the previously described subtypes, the entire GBV-C/HGV 5′ UTR from these samples was amplified and cloned; several clones from each sample (five clones from samples S2 and S4, four from S1 and S3, and one from S5) were sequenced. Comparison of evolutionary distances from all the clones examined revealed a genomic variability on two levels. One level was represented by the heterogeneity observed among the clones from each individual patient (Table II); the other one was represented by the variability among the isolates from different patients (mean distance: 0.0417; range: 0.0000–0.0751).

The results of the comparison of the evolutionary distances between all the clones examined from each patient and the 39 available sequences from GenBank are shown in Table III. In all cases, the GBV-C/HGV sequences were more related with subtype 2a than with the other subtypes. However, the evolutionary distances of all clones were not close enough to assume that these isolates could be classified as subtype 2a. The phylogenetic tree constructed with the nucleotide sequences showed that all clones obtained from these five patients segregated into a separate branch, which was different from those corresponding to the four reported subtypes (Fig. 2). Bootstrap analysis of 1,000 resamplings of the nucleotide sequences strongly supported the phylogenetic tree, as the sequences of the clones were grouped into a separate branch with a

TABLE III. Average Evolutionary Distances of the Whole GBV-C/HGV 5′ UTR Sequence Between All the Clones Analyzed in Each Patient and the Available 5′ UTR Sequences of Each Subtype

	GBV-C/HGV subtype[a]			
Sample	1	2a	2b	3
S1	0.1839 (0.1635–0.2019)	0.1022 (0.0860–0.1238)	0.1213 (0.1120–0.1351)	0.1333 (0.1275–0.1391)
S2	0.1824 (0.1591–0.2019)	0.0913 (0.0715–0.1007)	0.1093 (0.0969–0.1196)	0.1222 (0.1157–0.1272)
S3	0.1743 (0.1512–0.1937)	0.0922 (0.0715–0.1884)	0.1001 (0.0860–0.1084)	0.1036 (0.0934–0.1121)
S4	0.1702 (0.1358–0.1974)	0.0876 (0.0574–0.1123)	0.1052 (0.0788–0.1391)	0.1095 (0.0935–0.1273)
S5	0.2005 (0.1836–0.2190)	0.1091 (0.0969–0.1273)	0.1275 (0.1194–0.1349)	0.1330 (0.1311–0.1350)

[a]The numbers in parentheses indicate the range of the evolutionary distances.

bootstrap value of 70%. This result confirmed that these isolates could not be classified as subtype 2a.

No differences were observed in clinical features, sex, age, ALT levels, number of exposed donors, and diagnosis between the patients infected with subtype 2a or 2b and those infected with the strain of the undefined genotype (Table IV).

DISCUSSION

The existence of different GBV-C/HGV subtypes has been controversial. First, some researchers were able to identify GBV-C/HGV subtypes or subgroups based on the analysis of the 5′ UTR nucleotide sequence [Fukushi et al., 1996; Muerhoff et al., 1996, 1997; An et al., 1997]. However, others failed to find the same phylogenetic relationships when examining other regions of the GBV-C/HGV genome such as nonstructural region 3 (NS3) [Pickering et al., 1997], nonstructural region 5 (NS5) [Viazov et al., 1997], and the putative "core" peptide gene [Pickering et al., 1997]. Recently, Muerhoff et al. [1997] have demonstrated that small fragments of coding regions failed to reproduce the same groupings observed with the analysis of the 5′ UTR, although the results obtained with the 5′ UTR can be reproduced when the small fragments of coding regions are linked to form colinear segments [Muerhoff et al., 1997]. Thus, these researchers have identified four GBV-C/HGV subtypes whose prevalences differ in different areas.

In this study, the GBV-C/HGV subtype was determined in 16 Spanish multitransfused patients prior to BMT by comparing the evolutionary distances between our isolates and those from each subtype. According to this method, the GBV-C/HGV isolated from most patients belonged to subtype 2a, which is one of the predominant strains isolated in Europe and the United States [Muerhoff et al., 1996, 1997]. However, when the phylogenetic tree was constructed, this result could not be confirmed in all patients, since the isolates from five patients grouped into a separate branch that was different from the subtypes described previously but was closely related to the group 2 sequences. This result suggests that comparing the evolutionary distances is not enough to determine the GBV-C/HGV subtype infecting a given sample. To confirm the GBV-C/HGV subtype, the phylogenetic relationships between the nucleotide sequences from the samples and those from each subgroup must be established.

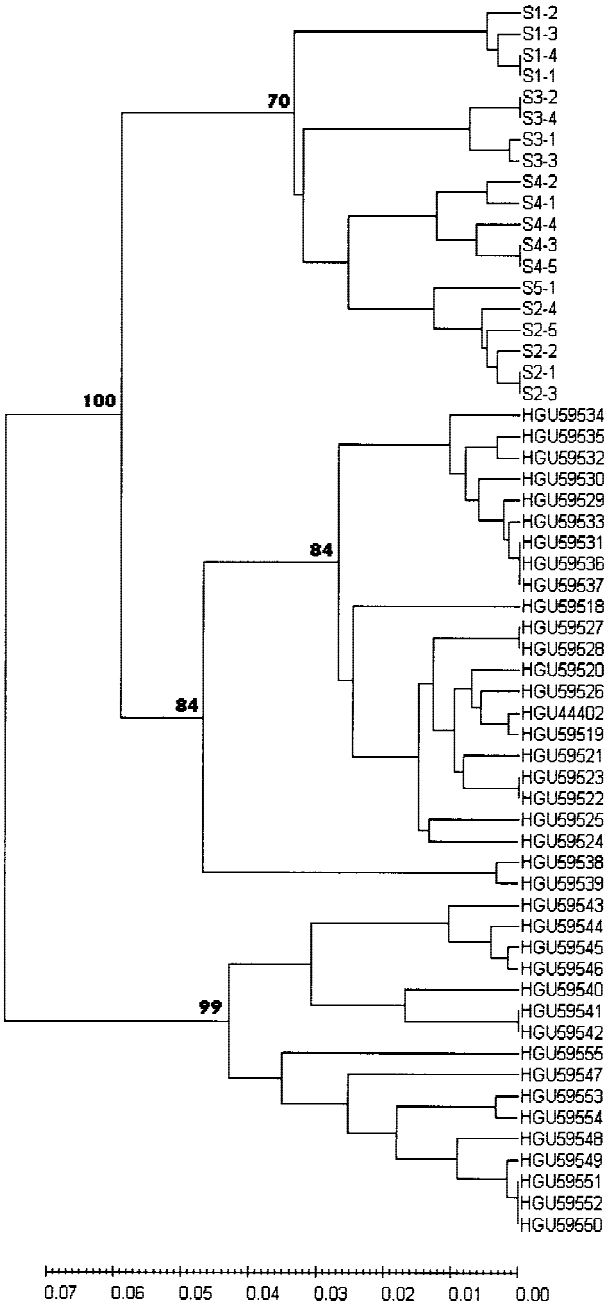

Fig. 2. Unrooted phylogenetic tree constructed with the entire GBV-C/HGV 5′ UTR sequences from the five patients with undefined subtype and the published sequences of each GBV-C/HGV subtype. Sample S1: clones S1-1, S1-2, S1-3, and S1-4; sample S2: clones S2-1, S2-2, S2-3, S2-4, and S2-5; sample S3: clones S3-1, S3-2, S3-3, and S3-4; sample S4: clones S4-1, S4-2, S4-3, S4-4, and S4-5; and sample S5: clone S5-1. The bootstrap values are indicated in the tree nodes.

TABLE IV. Clinical Features of Patients Infected With GBV-C/HGV Subtype 2a or 2b and Those Infected With the Undefined Subtype

	Subtype 2a or 2b	Undefined subtype
Number	11	5
Sex (male/female)	4/7	1/4
Age[a] (years)	34.4 ± 14.2	33.0 ± 13.9
ALT[a] (IU/l)	64.2 ± 65.3	33.4 ± 23.6
Diagnosis[b]		
AML	9	2
ALL	1	3
NHL	1	
Number of exposed donors[a]	109.9 ± 90.4	64.2 ± 63.6

[a]Expressed as the mean ± SD.
[b]AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma.

To ensure that the GBV-C/HGV isolated from these five samples with undefined subtype constituted a novel subtype, the whole 5′ UTR was cloned and sequenced. The genetic heterogeneity found among the different clones obtained from a single patient confirms the existence of GBV-C/HGV quasispecies, as has been previously reported by Viazov et al. [1997]. The quasispecies nature, which may influence the pathogenicity, virulence, and response to the therapy, has been described widely in other flaviviruses such as HCV [Bukh et al., 1995; Yeh et al., 1996; Maggi et al., 1997]. Although the GBV-C/HGV does not seem to play a role in causing chronic liver disease or to influence the outcome of BMT, further studies are needed to investigate the possible clinical and pathological implications of quasispecies in the course of infection.

In addition, the comparison of these isolates with the published sequences of each GBV-C/HGV subtype showed that they grouped into a new different branch. However, this branch appeared close to those of 2a and 2b sequences. Thus, this GBV-C/HGV sequence may be classified as genotype 2, but it represents a new subgroup within this group, which can be named tentatively subtype 2c. Whether this new subtype has any clinical implications in the setting of BMT remains to be studied further, although, at least in the short run, the clinical evolution of BMT in our patients infected with this subtype was similar to those infected with the other subtypes. Nevertheless, future studies in patients with BMT and in other types of patients will make it possible to assess the prevalence of this new subtype, to perform a more extensive phylogenetic analysis, and to study its possible geographical distribution and epidemiological implications.

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